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Polymerase chain reaction for detection of some highly dangerous viral fish disease agents

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SUMMARY
Today viral fish diseases cause major losses in the world aquaculture. Pathogen spread often occurs during the transportation of fish from infected farms to the disease-free ones. Therefore, the import of fish stocking material to Russia from countries with a different epidemic situation requires risk-based monitoring and forecasting. Diagnostics is of primary importance in the complex of measures to prevent the spread of viral infections in fish. To date, laboratory diagnostics of viral fish diseases is based on pathogen isolation and its identification using serological methods which require a lot of time and are performed only in large research institutes with specialized laboratories. Molecular diagnostic methods are more sensitive and high-performance. The article presents the results of using reverse transcription polymerase chain reaction to detect a number of highly dangerous viral diseases of fish (*Salmonidae*). As a result of this work, primers were selected and the temperature and time conditions of the reaction were optimized for the identification of infectious hematopoietic necrosis, viral hemorrhagic septicemia and infectious salmon anemia. The results obtained during the research allowed us to establish that this diagnostic method is highly specific with analytical sensitivity to infectious salmon anemia virus of 2.5 lg TCD_{50}/cm^3, to infectious hematopoietic necrosis – 2.9 lg TCD_{50}/cm^3 and to viral hemorrhagic septicemia – 4.2 lg TCD_{50}/cm^3. The described method was used to identify reference and field strains available at the FGBI ARRIAH Reference Laboratory for Aquaculture Diseases and isolated in different years in fish farms in the territory of the Russian Federation. The research data correlated with the results obtained from virus neutralization in cell culture and ELISA performed using commercial kits. The proposed method of RT-PCR allows to detect pathogens both in fish with pronounced clinical signs and in latent virus carriers.

Key words: fish diseases, infectious salmon anemia, viral hemorrhagic septicemia, infectious hematopoietic necrosis, reverse transcription polymerase chain reaction, specificity, analytical sensitivity.

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Применение полимеразной цепной реакции для выявления возбудителей некоторых особо опасных вирусных болезней рыб

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РЕЗЮМЕ
В настоящее время основной ущерб мировой аквакультуре наносят вирусные болезни рыб. Возбудители болезней часто проникают из одних рыбоводных хозяйств в другие при перевозках инфицированных рыб из неблагополучных хозяйств в благополучные. Поэтому завоз рыбопосадочного материала в Россию из стран с различной эпизоотологической ситуацией требует мониторинга и прогнозирования на основе возможных рисков. Ведущее место в комплексе мероприятий по предотвращению распространения вирусных инфекций рыб занимает диагностика. На сегодняшний день лабораторная диагностика болезней рыб вирусной природы основана на выделении возбудителя и его идентификации серологическими методами, которые требуют больших затрат времени и выполняются только в крупных научно-исследовательских институтах, имеющих профильные лаборатории. Более чувствительными и высокопроизводительными являются молекулярные методы диагностики. В статье представлены результаты применения полимеразной

цепной реакции с обратной транскрипцией по выявлению ряда особо опасных вирусных болезней рыб семейства лососевых. В результате проделанной работы были подобраны праймеры и оптимизированы температурно-временные условия проведения реакции для идентификации инфекционного некроза гемопоэтической ткани, вирусной геморрагической септицемии и инфекционной анемии лососевых. Результаты, полученные в ходе исследований, позволили установить, что данный диагностический метод является высокоспецифичным с аналитической чувствительностью для вируса инфекционной анемии лососевых – 2,5 lg $ТЦД_{50}/см^3$, инфекционного некроза гемопоэтической ткани – 2,9 lg $ТЦД_{50}/см^3$ и вирусной геморрагической септицемии – 4,2 lg $ТЦД_{50}/см^3$. С помощью описанного метода была проведена идентификация референтных и полевых штаммов, имеющихся в референтной лаборатории по болезням аквакультуры ФГБУ «ВНИИЗЖ» и выделенных в разные годы в рыбоводческих хозяйствах на территории Российской Федерации. Данные, полученные в ходе исследований, коррелировали с результатами вирусовыделения на культуре клеток и иммуноферментного анализа с использованием коммерческих наборов. Предложенная методика проведения полимеразной цепной реакции с обратной транскрипцией позволяет обнаружить возбудителей болезней у рыб как с явными клиническими признаками, так и со скрытым вирусоносительством.

Ключевые слова: болезни рыб, инфекционная анемия лососевых, вирусная геморрагическая септицемия, инфекционный некроз гемопоэтической ткани, полимеразная цепная реакция с обратной транскрипцией, специфичность, аналитическая чувствительность.

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INTRODUCTION

Today viral fish diseases have a world-wide distribution. About a quarter of the detected viruses cause diseases that result in serious losses in aquaculture. Among the most dangerous diseases are infectious hematopoietic necrosis, infectious salmon anemia, viral hemorrhagic septicemia, etc.[1] Despite the fact that most infections do not pose an immediate risk to human health, they have a negative impact on the fish growth rate, its marketable condition, the quality of fish products, and are accompanied by high mortality rates [1].

Disease prevention, i.e. prevention of pathogen introduction into disease-free regions, is the major tool for controlling infectious fish diseases all over the world. Therefore, the import of fish stocking material to Russia from countries with different epidemic situations requires risk-based monitoring and forecasting [2, 3, 4].

Diagnostics is of primary importance in preventing the spread of viral fish infections. To date, laboratory diagnosis of viral fish diseases is based on pathogen isolation and its identification using serological methods which require a lot of time and are performed only in large research institutes with specialized laboratories [1, 5].

Molecular diagnostic methods are more sensitive and require less time than culture and serological methods traditionally used to identify pathogens of viral fish diseases. Over the past decades, molecular diagnosis of diseases (including fish diseases) has advanced significantly. Such methods include polymerase chain reaction (PCR) which allows to detect the virus both in fish with pronounced clinical signs and in latent virus carriers[2].

In Russia there aren't any methods that are low cost, easy to use and, most importantly, high sensitive. Only imported kits and test systems for viral fish disease diagnosis are available on the domestic market.

The purpose of this study was to assess the possibility of using reverse transcription polymerase chain reaction (RT-PCR) for the detection and identification of highly dangerous viral diseases of fish (*Salmonidae*), such as viral hemorrhagic septicemia, infectious hematopoietic necrosis and infectious salmon anemia.

MATERIALS AND METHODS

The following strains were used in the study: Orenburg/14 strain of spring viraemia of carp virus (SVCV), Arkus 32/87 strain of infectious hematopoietic necrosis virus (IHNV), FLD/2004 strain of infectious pancreatic necrosis virus (INPV) and Aland strain of viral haemorrhagic septicaemia virus (VHSV), which are stored in the collection of microbial strains at the FGBI "ARRIAH", as well as CCBB strain of infectious salmon anemia virus (ISAV), obtained from the American Type Culture Collection (ATCC) in 2019.

The total RNA of the studied viruses was isolated using RNA-Extran commercial kit (Syntol, Russia) according to the recommendations of the manufacturer. The synthesis of cDNA from an RNA template was performed using OT-1 Test kit (Syntol, Russia), according to the recommendations of the manufacturer. The PCR was performed in a 25 µl reaction mixture using Encyclo Plus PCR kit (ZAO "Evrogen", Russia) according to the package insert. PCR was performed in a PTC-200 DNA Engine Cycler (Bio-Rad, USA). Previously published PCR primers were used [6, 7, 8]. The primers were synthesized by the Syntol company (Russia).

Amplicons obtained during PCR were analyzed by electrophoresis using 2% agarose gel and 10 mg/ml ethidium bromide in 1× trisborate buffer at 10 V/cm for one hour. PCR product (15 µl) was added to each well.

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M100 (5 µl; Syntol, Russia) was used as a molecular weight marker (100 bp increment). The gel was documentedusing Gel Doc XR+ Documentation System (Bio-Rad, USA).

The reaction sensitivity was analyzed by using ten-fold serial dilutions of viral cDNA. The analytical sensitivity limit was considered to be the highest dilution at which a positive result was registered.

RESULTS AND DISCUSSION

Based on the analysis of the studied viruses' genome sequences represented in the GenBank database of the U.S. National Center for Biotechnology Information (NCBI), and the recommendations of the World Organization for Animal Health (OIE), primers specific for conserved regions of the eighth segment of the ISA virus, of nucleocapsid (N) gene of VHSV and of G gene of IHNV, allowing to identify the maximum number of known strains and isolates of these viruses, were selected. The primer sequences are presented in Table 1.

During a series of experiments, temperature and time conditions for PCR were optimized. When amplifying a fragment of the IHN virus genome, the number of cycles was increased from 30 to 35, which allowed a larger number of amplicons to be produced, thereby increasing the sensitivity of the reaction. When amplifying a fragment of the ISA virus genome, the annealing time was reduced from 45 to 30 seconds, and the synthesis time was reduced from 90 to 60 seconds, which allowed to reduce the reaction time without losing its effectiveness. When amplifying a fragment of the VHS virus genome, the annealing temperature was increased from 52 to 55 °C, which increased the specificity of the reaction.

The optimized amplification parameters for the studied infections are presented in Table 2.

The specificity of the selected primers was tested experimentally during the study of cultures inoculated with SVC, VHS, IPN, IHN and ISA viruses using RT-PCR. The virus genome fragments obtained after amplification were analyzed using agarose gel. The gel was examined under UV-light (wavelength 312 nm) and the reaction results were interpreted based on the presence or absence of luminous bands.

Figure 1 shows the electrophoregrams of the analysis.

From the data presented in Figure 1, it can be seen that for wells No. 2 containing IHN, ISA and VHS virus strains, clear fragments are observed at 693 bp (*a*), 211 bp (*b*) and 811 bp (*c*), respectively. As for the other wells, including the ones with negative control, these fragments are not observed, which indicates the high specificity of the selected primers for detecting the pathogens causing the above-mentioned infections and the absence of contamination of the used reagents of the tested or foreign RNA.

Analytical sensitivity was estimated on ten-fold serial dilutions of cDNA obtained from reverse transcription with the RNA extracted from ISA, VHS and IHN virus cultures which had initial infectivity titres of 5.5; 7.2 and 6.9 lg TCD_{50}/cm^3, respectively. The sensitivity limit was considered to be the highest dilution at which a positive result was registered. The calculated values of the analytical sensitivity of the optimized PCR were 2.5 lg TCD_{50}/cm^3 for the ISAV, 2.9 lg TCD_{50}/cm^3 for the IHNV, and 4.2 lg $TCD_{50}/см^3$ for the VHSV.

The results of analytical sensitivity testing are provided in Figure 2 and Table 3.

Table 1
Design of primers for detection of pathogens causing infectious salmon anemia, viral hemorrhagic septicemia and infectious hematopoietic necrosis

Таблица 1
Структура праймеров для выявления возбудителей инфекционной анемии лососевых, вирусной геморрагической септицемии и инфекционного некроза гемопоэтической ткани

Primer sequence (5′–3′)	Size of PCR-product, bp	Reference
ISAV		
F-GAAGAGTCAGGATGCCAAGACG	211	M. Devold et al. [6]
R-GAAGTCGATGAACTGCAGCGA		
VHSV		
F – GGGGACCCCAGACTGT	811	OIE. Manual of Diagnostic Tests for Aquatic Animals [8]
R-TCTCTGTCACCTTGATCC		
IHNV		
F – AGAGATCCCTACACCAGAGAC	693	E. J. Emmenegger et al. [7]
R – GGTGGTGTTGTTTCCGTGCAA		

F – forward primer;
R – reverse primer.

Table 2
Amplification parameters

Таблица 2
Параметрыамплификации

PCR stage	Number of cycles	Temperature, °C	Stage duration, min
Pre-denaturation	1	94	3
Denaturation	35	94	1/2
Annealing of primers		59 (ISA) 50 (IHN) 55 (VHS)	1/2
Synthesis		72	1
Final synthesis	1	72	7
Storage	1	4	20

Thus, the proposed RT-PCR method allows to detect ISA, IHN and VHS viruses at their minimum infectivity titres of 2.5; 2.9 and 4.2 lg $TCD_{50}/см^3$, respectively. The specificity of the selected primers was tested using the BLAST program, as well as experimentally. The PCR results presented in Figure 1 show that the selected primers hybridize only with the fragments complementary to the target viral RNA and do not interact with the RNA of nontargets.

The described method was used to identify reference and field strains isolated on fish farms in the territory of the Russian Federation in different years and available at the FGBI "ARRIAH" Reference Laboratory for Aquaculture

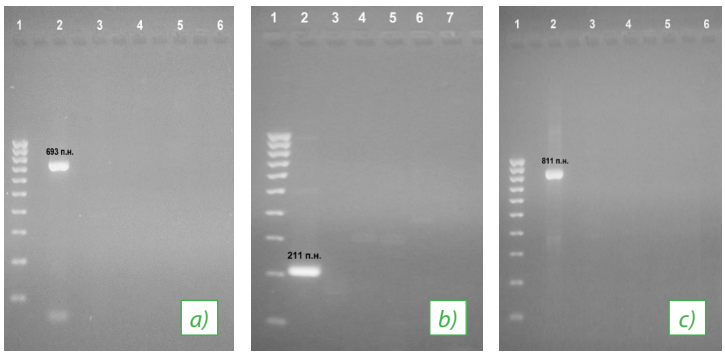

Fig. 1. Electrophoregrams of RT-PCR specificity testing results for detection of infectious hematopoietic necrosis (IHN) (a), infectious salmon anemia (b) and viral hemorrhagic septicemia (c) viruses

a) 1 – molecular weight markers "M100";
2 – Arcus 32/87 strain of the IHN virus;
3 – Aland strain of the viral hemorrhagic septicemia (VHS) virus;
4 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
5 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
6 – negative control (sterile water free of RNase and DNase);
b) 1 – molecular weight markers "M100";
2 – CCBB strain of the infectious salmon anemia (ISA) virus;
3 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
4 – Aland strain of the VHS virus;
5 – Arcus 32/87 strain of the IHN virus;
6 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
7 – negative control (sterile water free of RNase and DNase);
c) 1 – molecular weight markers "M100";
2 – Aland strain of the VHS virus;
3 – Arcus 32/87 strain of the IHN virus;
4 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
5 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
6 – negative control (sterile water free of RNase and DNase).

Рис. 1. Электрофореграммы результатов тестирования специфичности метода ОТ-ПЦР для выявления вирусов ИНГТ (a), ИАЛ (b) и ВГС (c)

a) 1 – маркер молекулярных весов «M100»;
2 – штамм «Аркус 32/87» вируса ИНГТ;
3 – штамм «Аланд» вируса ВГС;
4 – штамм «FLD/2004» вируса ИНПЖ;
5 – штамм «Оренбург/14» вируса ВВК;
6 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);
b) 1 – маркер молекулярных весов «M100»;
2 – штамм «ССВВ» вируса ИАЛ;
3 – штамм «Оренбург/14» вируса ВВК;
4 – штамм «Аланд» вируса ВГС;
5 – штамм «Аркус 32/87» вируса ИНГТ;
6 – штамм «FLD/2004» вируса ИНПЖ;
7 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);
c) 1 – маркер молекулярных весов «M100»;
2 – штамм «Аланд» вируса ВГС;
3 – штамм «Аркус 32/87» вируса ИНГТ;
4 – штамм «FLD/2004» вируса ИНПЖ;
5 – штамм «Оренбург/14» вируса ВВК;
6 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз).

Diseases. The data obtained in the studies using RT-PCR correlated with the results obtained by virus isolation in cell culture and by ELISA using commercial kits produced by TestLine Clinical Diagnostics Ltd. (Czech Republic) and Bio-X Diagnostics S.A. (Belgium).

Table 3
Determination of optimized RT-PCR analytical sensitivity

Таблица 3
Результаты определения аналитической чувствительности оптимизированной ОТ-ПЦР

No.	Dilution	Virus infectivity titre, Lg TCD_{50}/см^3	RT-PCR result
		ISAV	
1	original sample	5.5	+
2	10^{-1}	4.5	+
3	10^{-2}	3.5	+
4	10^{-3}	2.5	+
5	10^{-4}	1.5	–
6	10^{-5}	0.5	–
		IHNV	
1	original sample	6.9	+
2	10^{-1}	5.9	+
3	10^{-2}	4.9	+
4	10^{-3}	3.9	+
5	10^{-4}	2.9	+
6	10^{-5}	1.9	–
		VHSV	
1	original sample	7.2	+
2	10^{-1}	6.2	+
3	10^{-2}	5.2	+
4	10^{-3}	4.2	+
5	10^{-4}	3.2	–
6	10^{-5}	2.2	–

"+" – positive result;
"–" – negative result.

The results presented in the article on the use of conventional gel-based RT-PCR show that despite the high effectiveness of this method, it is still inferior to more modern molecular genetic diagnostic methods, such as real-time RT-PCR and microchip-based RT-PCR. The main advantages of the latter two methods: they are more sensitive, less time consuming and there is reduced contamination due to the absence of the gel electrophoresis. Despite a number of advantages, real time RT-PCR is not widely used in our country for viral fish disease diagnosis and was used only by M. I. Doronin et al. together with the employees of the company OOO "Lumex-marketing" (Saint Petersburg) for IHNV and VHSV detection [9].

It should be mentioned that in Russia data on the use of RT-PCR for diagnosis of such viral fish diseases as IHN, VHS and ISA is scarce. There is a limited number of publications on the development and use of this molecular genetic method [2, 9, 10, 11], while it has become commonly used abroad. In many countries, selective amplification of viral genome fragments using PCR with subsequent sequencing is used not only for the diagnosis of IHN, VHS and ISA viruses, but also for the development of methods for their typing [12, 13, 14].

CONCLUSION

As a result of the research, primers were selected and the temperature and time conditions of PCR were optimized to detect such viral fish diseases as viral hemorrhagic septicemia, infectious hematopoietic necrosis and infectious salmon anemia.

The results demonstrate high specificity of this diagnostic method and its analytical sensitivity of 2.5 lgTCD_{50}/см^3 when detecting infectious salmon anemia virus, 2.9 lg TCD_{50}/см^3 – infectious hematopoietic necrosis virus and 4.2 lg TCD_{50}/см^3 – viral hemorrhagic septicemia virus. The RT-PCR allowed to reduce testing time to 5 hours, while the "gold" standard – virus isolation – takes several weeks.

The proposed method can be used to detect ISA, IHN and VHS viruses in monitoring studies of pathological material samples collected from salmonid fishes. The peculiarity of this method is that it allows to detect infected fish before the onset of clinical signs, in latent virus carries, which is especially important for cross-border transportation of hydrobionts.

Upon the results of the studies, guidelines for ISA, IHN and VHS virus identification using gel-based RT-PCR were prepared and approved by the Scientific Council of the FGBI "ARRIAH".

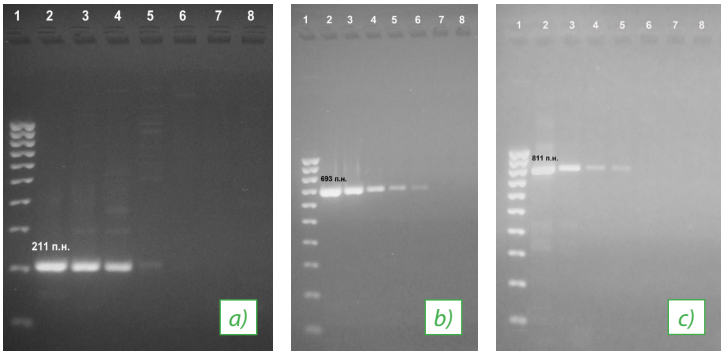

Fig. 2. Electrophoregrams showing PCR analytical sensitivity testing results for detection of ISA (a), IHN (b) and VHS (b) viruses

a) *1 – molecular weight markers "M100";*
2 – original sample (CCBB strain of the ISA virus);
3–7 – a series of ten-fold dilutions of CCBB strain of the ISA virus (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase);

b) 1 – molecular weight markers "M100";
2 – original sample (Arcus 32/87 strain of the IHN virus);
3–7 – a series of ten-fold dilutions of Arcus 32/87 strain of the IHNV (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase);

c) 1 – molecular weight markers "M100";
2 – original sample (Aland strain of the VHS virus);
3–7 – a series of ten-fold dilutions of Aland strain of the VHS virus (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase).

Рис. 2. Электрофореграммы результатов тестирования аналитической чувствительности ПЦР для выявления вирусов ИАЛ (a), ИНГТ (b) и ВГС (c)

a) 1 – molecular weight markers «M100»;
2 – исходный образец (штамм «CCBB» вируса ИАЛ);
3–7 – серия десятикратных разведений штамма «CCBB» вируса ИАЛ (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);

b) 1 – molecular weight markers «M100»;
2 – исходный образец (штамм «Аркус 32/87» вируса ИНГТ);
3–7 – серия десятикратных разведений штамма «Аркус 32/87» вируса ИНГТ (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);

c) 1 - molecular weight markers «M100»;
2 – исходный образец (штамм «Аланд» вируса ВГС);
3–7 – серия десятикратных разведений штамма «Аланд» вируса ВГС (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз).

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